F ..m C. H. BROWNING, M.D., LL.D., D.P.H., F.R.S.,
PROFESSOR OF BACTERIOLOGY,
UNIVERSITY OF GLASGOW.

Department of Bacteriology,
THE UNIVERSITY,
GLASGOW, W.2
TEL. WESTERN 3981.

12th June, 1950

Dr. J.Lederberg,
Department of Genetics,
College of Agriculture,
University of Wisconsin,
Madison, Wisconsin.

Dear Dr. Lederberg,

I have read with much pleasure your work in J. Bacteriology, 1950 59, 211. In reference to your suggestion that "recombination might possibly play some role in the establishment of multiply resistant bacteria, under the influence of chemotherapy", I trust that this line will develop fruitfully and shall be glad to get reprints. You will be interested to know that in the case of drug-resistant trypanosomes I carried out similar experiments, but unfortunately with negative results so far as evidence of recombination was concerned - see p.181 of Browning, J. Path. & Bact., 1908, 12, 166.

Yours sincerely,

C. H. Browning

CHEMO-THERAPY IN TRYPANOSOME-INFECTIONS: AN EXPERIMENTAL STUDY.[1]

By CARL H. BROWNING, M.D., *Assistant in Bacteriology to the Professor of Pathology, Glasgow University; formerly Carnegie Research Fellow.*

From the Königliches Institut für experimentelle Therapie and the Georg Speyer Haus, Frankfurt a. Main, Director Geheimrath Prof. Ehrlich.

THE wide distribution of diseases due to trypanosomes, and the gravity of the results to which they give rise, make the experimental study of the action of drugs in infections with these pathogenic agents of practical as well as of theoretical interest. It is in this group of diseases, too, that the most promising results have been obtained by the use of chemo-therapeutic agents. The first work in this direction was that of Laveran and Mesnil (11. 1902). They showed that arsenite of soda had a marked, though only temporary, beneficial effect on the course of nagana-infections in rats and mice. The discovery of trypan-red by Ehrlich (1904) along with Shiga and Weinberg, marked the first stage of progress in the search for synthetic compounds which will, in the body of an infected animal, exert a specific antagonism toward the pathogenic agent. In the case of mice infected with the trypanosomes of mal de Caderas, a single injection of trypan-red leads to the destruction of all the parasites, and so definitely cures what is, in untreated animals, an acute and certainly fatal disease. Elsewhere (1907) I have dealt with the later phases of investigation on the chemo-therapy of trypanosomiases, and have discussed the bearing of the facts on questions of treatment of the naturally occurring disease. Accordingly, the present paper is concerned with—(1) a study of the action of some of the principal chemo-therapeutic agents which are effective in experimental trypanosomiases in mice, especially the para-amido-phenylarsenic-acid-derivatives of Ehrlich; (2) the development of trypanosome-strains "resistant" to chemo-therapeutic agents of different types, and an examination of their further biological properties; (3) the phenomena of immunity after complete or almost complete destruction of the parasites by means of chemo-therapeutic agents.

[1] The work here published forms part of a thesis for the degree of M.D. in the University of Glasgow, and was carried out during the tenure of a Carnegie Fellowship.
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Methods and Details of the Experimental Infections.

White mice were employed. Each animal was inoculated by subcutaneous injection with 1 c.c. of a 2 per cent. dilution in 0·85 per cent. NaCl solution of blood containing abundant parasites taken from another mouse at a period within twenty-four hours of death in the case of nagana, or twenty-four to forty-eight hours in dourine-infections. The course of the infection can be schematically represented as follows (Table I):—

Table I.

	Nagana.		Dourine.	
	A.	**B.**	**A.**	**B.**
First day after inoculation . . .	+	+	–	+
Second ,, ,, ...	+ +	+ + +	+	+
Third ,, ,, . . .	Dead	Dead	+ +	+ +
Fourth ,, ,, . . .	...	...	+ + +	+ + +
Fifth ,, ,, . . .	...	...	Dead	+ + +
Sixth ,, ,, . . .	...	...	..	+ + +
Seventh ,, ,, . . .	...	...	...	Dead

In this and the following tables—

– = No parasites.

\+ = Up to half a dozen parasites per field in a fresh wet film of blood (from the tail) one corpuscle in thickness, with a Zeiss "D" lens and No. 2 ocular.

\+ + = Ten to twenty parasites per field.

\+ + + = Innumerable parasites.

The parasites increase progressively in the blood. In nagana-infections death invariably occurs on the third day; and in dourine, from two to four days later. For the therapeutic experiments this standard inoculation was always used; but a number of observations has been made on the effect of varying the dose of the inoculation-material in the case of nagana. The results of three such series are given in Table II. All the mice in series A were inoculated with the same infective material, the only difference being in the amount, and so also in B and in C. Thus, it appears that the duration of the disease depends to some extent on the dose, very large amounts (0·2 c.c.) causing death within thirty-six hours (*vide* series A). The injection of 0·005 c.c.—only a fourth of the standard dose—produces an infection which is little if at all protracted. In the case of extreme dilutions (*vide* series B and C) the incubation-period is prolonged; but after the appearance of scanty parasites the infection, as a rule, follows the usual course. In two instances infection did not occur after minute doses (0·0001 and 0·00001 c.c.). This is, in all probability, an indication of a degree of natural resistance to infection which varies with the individual, and which can come into play effectively only where a very small number of parasites is injected. That such a resistance does appear when attenuated strains are used for inoculation will be shown later.

TABLE II.

A.

Amount of Blood used for Inoculation.	0·2 c.c.	0·05 c.c.	0·01 c.c.	0·005 c.c.
First day after inoculation . . .	+ +	+ +	+	+
Second ,, ,, . . .	Dead 1½ days	+ + +	+ + +	+ +
Third ,, ,, . . .	...	Dead 2¼ days	Dead	Dead

B.

Amount of Blood used for Inoculation.	0·005 c.c.		0·0005 c.c.			0·00005 c.c.		
	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
First day after inoculation .	−	−	−	−	−	−	−	−
Second ,, ,, .	+ +	+ +	−	−	+	−	−	−
Third ,, ,, .	+ + +	+ + +	+	+	+ +	−	−	+
Fourth ,, ,, .	Dead	Dead	+ + +	+ + +	+ + +	+	+	+
Fifth ,, ,, .	...	...	Dead	Dead	Dead	+ + +	+ + +	+ + +
Sixth ,, ,, .	...	...	...	...	...	Dead	Dead	Dead
Seventh ,, ,, .	...	...	...	...	...	...	...	...

	0·000005 c.c.			0·0000005 c.c.		
	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
First day after inoculation .	−	−	−	−	−	−
Second ,, ,, .	−	−	−	−	−	−
Third ,, ,, .	−	−	−	−	−	−
Fourth ,, ,, .	−	−	−	−	−	+
Fifth ,, ,, .	+	+	+	+	+	+ +
Sixth ,, ,, .	+ + +	+ + +	Dead	+ + +	+ + +	+ + +
Seventh ,, ,, .	Dead	Dead	...	Dead	Dead	Dead

C.

Amount of Blood used for Inoculation.	0·01 c.c.	0·001 c.c.			0·0001 c.c.		0·00001 c.c.		
		(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)
First day after inoculation	−	−	−	−	−	−	−	−	−
Second ,, ,,	+	−	−	−	−	−	−	−	−
Third ,, ,,	+ + +	+	+	+	−	−	−	−	−
Fourth ,, ,,	Dead	+ + +	+ + +	+ + +	−	−	−	−	−
Fifth ,, ,,	...	Dead	Dead	Dead	+	−	−	+	+
Sixth ,, ,,	...	...	...	...	+ +	−	−	+ +	+ +
Seventh ,, ,,	...	...	...	...	dead	−	−	dead	dead
Thirty-sixth ,, ,,	...	...	...	...	...	Dead	−	...	...
Ninety-first ,, ,,	...	...	...	...	...	Never became infected	...	...	

In regard to the employment of chemo-therapeutic substances there are three general considerations—(1) dosage and mode of administration; (2) the time of intervention in the course of the infection; (3) the criterion of effective treatment. As to *dosage*: even the most efficient substances known are certain in their action only when the dose approaches the toxic limit, and a proportion of hyper-sensitive animals succumb to the treatment. Accordingly, the "therapeutic dose" is fixed at two-thirds of the maximum dose which average animals will tolerate The high doses employed by some workers would indicate that different strains of mice vary in their susceptibility to toxic action. The hyper-sensibility of particular individuals is independent of weight. The *mode of administration*: (1) Subcutaneous injection. In referring to the dosage the very convenient arrangement introduced by Ehrlich will be used throughout. The statement that "the therapeutic dose is 1 : 100" means that the dose given to a mouse weighing 20 grms. is 1 c.c. of a 1 per cent. solution; on this basis a 15-grm. mouse gets 0·75 c.c., and so on. (2) The method of feeding with the substance incorporated in "cakes," first employed by Ehrlich. This is of great value where compounds, *e.g.* the basic triphenylmethane dyes, produce extensive induration and necrosis when injected, but are readily resorbed from the alimentary canal, and also where it is desired to keep the body saturated with a drug for a long period. *The time of intervention* "under standard conditions" in nagana-infections has always been twenty-four hours after inoculation, when scanty parasites are already found in the blood on microscopic examination. As will be seen later, the efficiency of a chemo-therapeutic agent, other things being equal, depends on the time which has elapsed between inoculution and treatment. The *criterion of sterilisation* and cure is, of course, that the parasites never again appear in the blood. In the case of the very virulent nagana-strain used in this work, the treatment being carried out under standard conditions, it has been judged sufficient to examine the blood daily for the first five weeks after treatment, then at intervals for the next ten days, and thereafter to keep the animals under observation in the laboratory for at least three months. If at the end of this time the blood is still free of parasites the animal is counted as "cured." In no instance has an animal died from trypanosome-infection during the latter three months. Where treatment was begun on the second day after inoculation, and where recurrences were treated, no animal was held to be cured unless its blood remained free of parasites for six months after cessation of treatment.

I. The Action of Chemo-Therapeutic Agents in Experimental Trypanosome-Infections.

The effective compounds, so far as is at present known, belong to three distinct chemical groups—(1) dyes which are derivatives of

benzidin and its analogues; (2) basic triphenylmethane dyes; (3) arsenical compounds, especially atoxyl, which is the sodium salt of para-amido-phenylarsenic-acid and its derivatives. Table III. shows the therapeutic doses of the compounds employed.

1. *Benzidin Dyes.*

Trypan-red (1 molecule tetrazotised benzidin-monosulphonic acid + 2 molecules Na-naphthylamin-disulphonate 2, 3, 6) seldom produces complete cure in nagana-infections.

Trypan-blue (1 molecule tetrazotised tolidin + 2 molecules Na-amido-naphthol-disulphonate 1, 8, 3, 6) was introduced by Mesnil and Nicolle (1906). It is much more effective than trypan-red in nagana-infections, and a complete cure occurs in a number of instances.

TABLE III.

Compound.	Dose.
Trypan-red	1 : 200
Trypan-blue	1 : 200
Trypan-violet	1 : 150
Parafuchsin	1 : 1000
Ethyl-violet	1 : 750
Döbner's violet	1 : 500
Atoxyl	1 : 300
Acetyl-para-amido-phenylarsenic acid and the par-oxy-benzyliden-derivative	1 : 30

Trypan-violet (1 molecule para-diamido-diphenylurea + 2 molecules Na-amido-naphthol-disulphonate 1, 8, 3, 6)—Mesnil and Nicolle—is not much more efficient than trypan-red in nagana-infections.

After injections of all these drugs the skin becomes dyed, and the colour persists for several months. They have practically no trypanocidal action *in vitro*.

2. *Basic Triphenylmethane Dyes.*

Parafuchsin (triamido-triphenylmethane-chlorhydrate) was introduced by Ehrlich and Franke (1907). The therapeutic dose, under standard conditions, both in nagana-infections and in dourine, causes the parasites to disappear from the blood for from seven to twelve days; but very rarely causes complete cure. Induration and necrosis frequently follow subcutaneous injection. Ehrlich found that parafuchsin oleate is well resorbed from the alimentary canal. Powdered "Albert" biscuit is soaked in a solution of 1 grm. parafuchsin base dissolved by heat in 10 grms. oleic acid 1 (Kahlbaum), and 90 grms. alcohol, in the proportion of 3 c.c. to each biscuit weighing 8 grms. When the alcohol has evaporated, 0·6 of a grm. of "glidin" per biscuit is added; a dough is made with milk, rolled out thin and allowed to dry.

Mice will tolerate continuous feeding with these cakes for months. Animals fed for some days and then inoculated with nagana and fed further for a week almost never become infected (Ehrlich (1907)). In experiment of this kind, performed by the author, eleven mice were fed with parafuchsin for six days before inoculation, and for from seven to nine days thereafter; ten of these mice never became infected. When the parafuchsin-feeding is not begun till parasites have appeared in the blood recurrence is the rule.

Ethyl-violet (hexa-ethyl-pararosanilin) is less efficient than parafuchsin in nagana-infections.

Döbner's violet (diamido-triphenylmethane-chlorhydrate) approaches parafuchsin in its action in nagana-infections.

These compounds do not cause the skin to become dyed; they are actively trypanocidal *in vitro*.

3. *Arsenical Compounds.*

Arsenious acid injected at the same time as the inoculation with nagana frequently did not cause even twenty-four hours' protraction of the infection.

Atoxyl was first of all introduced as a therapeutic agent in the treatment of trypanosome-infections by Thomas (1905). Ehrlich and Bertheim (1907) have determined its constitution. It is a sodium salt of para-amido-phenylarsenic acid, $C_6H_4\begin{cases}NH_2\ (1)\\AsO(OH)_2\ (4),\end{cases}$ and contains 24·1 per cent. of arsenic. Ehrlich and the author have investigated the toxicity of this substance in an extensive series of animals. With the strains of mice in their hands $\frac{1}{300}$ grm. was the largest dose which was tolerated, on the average, by a 20-grm. mouse. But some animals will tolerate double this amount. Of eleven mice receiving a dose of 1 : 200, seven survived and four died. Of twenty-nine mice receiving 1 : 150, only seven survived; of these only one was distinctly ill, the others showed little or no sign of intoxication. Death does not occur at once; five died on the third, seven on the fourth, five on the fifth day. Animals which have once borne a large dose (1 : 150) will again, after an interval of ten days, bear a repetition of the same injection. On the other hand, particular, hyper-sensitive, animals die four or five days after a single dose of 1 : 300. Accordingly, Ehrlich concludes that each animal has tolerably stable constitutional characters which determine its susceptibility ("gift-titer"). The results of the author's experiments with atoxyl in nagana-infections under standard conditions are shown in Table IV. Thus, in the majority of instances the therapeutic dose

TABLE IV.

Dose of Atoxyl.	Results.				Total.
	Cured.	Recurrences.	Blood never rendered free of Parasites.	Animals dead from intoxication; Blood free of Parasites.	
1 : 200 to 1 : 250 .	1	8	3	10	22
1 : 300 . . .	5	30	16	18	69
1 : 350 to 1 : 450 .	0	8	5	1	14

(1 : 300) renders the blood free of parasites for a period up to fourteen days. Frequently, however, the trypanosomes never disappear from the blood. Very commonly, animals whose blood has been rendered free die poisoned four or five days after treatment. The great irregularity of the action of atoxyl is well shown in the series in Table V. Cakes containing 0·125 per cent. by weight of atoxyl are tolerated usually for ten consecutive days. Feeding begun twenty-four hours after inoculation and continued for three days after disappearance of parasites from the blood leads to cure not infrequently (Röhl).

TABLE V.

Serial No.	W. 16.	W. 17.	W. 18.	W. 19.	Controls.	
					(*a*)	(*b*)
1 day after inoculation .	Inf. +[1]	Inf. +[1]	Inf. +[1]	Inf. +[1]	Inf. +	Inf. +
2 ,, ,,	+	–	–	+	+ + +	+ + +
3 ,, ,,	+	–	–	–	Dead	Dead
4 ,, ,,	+ + +	–	– Ill	–	...	...
5 ,, ,,	Dead	–	Dead (poisoned)	–	...	...
6 to 8 ,, ,,	...	–	...	–	...	...
9 ,, ,,	...	–	...	+	...	...
10 ,, ,,	...	–	...	+ + +	...	...
11 ,, ,,	...	–	...	Dead.	...	...
156 ,, ,,	...	Cured	...	...	...	...

[1] = Atoxyl 1 : 300 injected subcutaneously.

Acetyl-para-amido-phenylarsenic-acid and the *Para-oxybenzylidene-derivative of para-amido-phenylarsenic-acid* introduced by Ehrlich are the most efficient agents in the treatment of nagana-infections in mice at present known. The acetyl-derivative contains 29 per cent. of arsenic, and the para-oxybenzylidene-compound 24 per cent. More than ten times as much of these compounds as of atoxyl can be borne. A dose of 1 : 20 is frequently tolerated, and 1 : 30 to 1 : 40 can be injected on five or even more consecutive days. A remarkable series of symptoms often follows the administration to mice of the acetyl-derivative, in the form either of a single therapeutic dose, or of repeated smaller doses, or after feeding, and also after injections of the para-oxybenzylidene-derivative. On the day after a single large injection the animal usually, when undisturbed, sits quietly in its glass; but when it moves a general muscular tremor becomes apparent. This is very marked in the case of the neck-muscles, the head is thrown about

in all directions; especially characteristic is a jerking hyper-extension of the head, causing the animals to look up into the air. Three or four days later the co-ordination of the limb-movements is seen to be disturbed. The mouse cannot run forward, but exhibits a distinct retrogression, the hind legs being spread wide apart. After a time the retrogression-movement becomes less apparent, and then the mice begin to behave in a manner indistinguishable from that of Japanese dancing-mice. In their glasses or on the open floor they go round in circles for hours at a stretch. The animals turn indifferently in one direction or the other; but each seems to "prefer" one direction, making only occasionally a few reverse turns. This condition has persisted undiminished in animals under observation for twelve months. Otherwise, the animals are in excellent condition, and eat much more than normal mice. Mesnil, Nicolle, and Aubert (1. 1907) have recently described a similar condition in rats treated with atoxyl; this has also been observed by the author; but the phenomenon was not so pronounced as in the mice treated with the derivatives.

In test-tube experiments the sodium salt of acetyl-para-amido-phenylarsenic acid in water, in a concentration equal to 1 per cent. of the free acid, has no effect on the mobility of the trypanosomes of nagana after contact for two hours and even longer at room-temperature. Under the same conditions a 1 per cent. solution of atoxyl shows only a very weak effect on the trypanosomes. The results of therapeutic experiments with these derivatives, under standard conditions, is given in Table VI. It is seen that—(1) a single therapeutic dose has effected complete cure in 94 per cent. of the animals which tolerated the injection; (2) a half to a fifth of the therapeutic dose rendered the blood free in every case, and cured 36 per cent.; (3) doses so small as a seventh to a tenth of the therapeutic dose were not much inferior to the therapeutic dose of atoxyl. Equally good results were got in dourine-infections.

TABLE VI.

Dose.	Results.				Total.
	Cured.	Recurrences.	Blood never rendered free of Parasites.	Animals dead from intoxication; Blood free of Parasites.	
1 : 30 to 1 : 50 . .	31	2	0	3	36
1 : 60 to 1 : 150. .	8	14	0	0	22
1 : 200 to 1 : 300 .	0	7	0	1 (?)	8

The next point to be determined was the *effect of giving the therapeutic injection on the second day after inoculation* with nagana, *i.e.* at a time within twelve to eighteen hours before death, when the blood is swarming with parasites.

Twelve mice received a single dose (1 : 40 to 1 : 50) of the para-oxybenzylidene-compound under these conditions. In every case the blood was found free of trypanosomes on microscopic examination twenty-four hours later; but in every instance, after a period of from ten to seventeen days, recurrence took place, and the infection terminated fatally. In five animals treated with 1 : 75, recurrence took place seven to twelve days later. As a control, a number of animals of the same series were treated with atoxyl on the second day; five which received a large dose (1 : 200 to 1 : 250) had recurrences from the seventh to the thirteenth day; and of five receiving the therapeutic dose (1 : 300) the blood in three was never rendered free, and the other two had recurrences on the seventh day. The treatment of advanced infections by repeated small doses was also unsatisfactory. Out of seventeen animals which received from three to eight injections of the para-oxybenzylidene-compound 1 : 70, only five were cured. Thus, it is clear that neither a single large dose nor repeated small ones sufficed. Accordingly, in two series, three to five injections of the acetyl-derivative in a dose of 1 : 40 were given. In some the first three doses were given on consecutive days; in others only the first two. Table VII. gives a representative example of the proceeding. Of twenty-two animals, 12 were cured (55 per cent.); five died with their blood free of parasites; five had a recurrence.

TABLE VII.

Serial No. of Mouse.	No. of Injections.	Days subsequent to Inoculation on which Injections were given.	Result.
Y 100 . . .	5	2, 3, 8, 16, 24	Cured
Y 101 . . .	3	2, 3, 8	Recurrence sixteen days after last injection
Y 102 . . .	3	2, 3, 8	Died ten days after last Injection. Blood free of parasites
Y 103 . . .	3	2, 3, 16	Cured

These results show clearly that the difficulty in effecting cure increases with the duration of the infection; or, in other words, with the number of parasites in the blood. Of course, it must be remembered that when the infection is advanced the animals are in a highly abnormal state.

In such advanced infections the method of *combined treatment*, where compounds belonging to different chemical groups are used in alternation, gives much better results than injections of any one compound. The facts which establish the theoretical correctness of this procedure will be discussed later. By the use of trypan-blue in alternation with the acetyl-derivative twenty-three out of thirty-three animals, *i.e.* sixty-nine per cent., were cured. Table VIII. illustrates the mode of procedure.

TABLE VIII.

Serial number of Mouse.	Therapeutic Agent and Dose.	Number of Injections.	Days subsequent to Inoculation on which Injections were made.	Result.
Y 105 . .	Acetyl-derivative, 1 : 40	4	2, 3, 8, 21	Cured
	Trypan-blue, 1 : 200	1	4	
Y 106 . .	Acetyl-derivative, 1 : 40	4	2, 3, 8, 16	Recurrence ten days after the last injection
	Trypan-blue, 1 : 200	1	4	
Z 65 . .	Acetyl-derivative, 1 : 40	4	2, 3, 4, 8	Cured
	Trypan-blue, 1 : 200	1	10	
Z 66 . .	Acetyl-derivative, 1 : 40	4	2, 3, 4, 8	,,
	Trypan-blue, 1 : 200	1	10	

Specially favourable results have been got in advanced infections by an injection of the acetyl-derivative 1 : 40, on the second day after inoculation, followed by feeding with cakes containing 0·125 per cent. of atoxyl, begun on the next day, and continued for ten or eleven days. Of eleven animals thus treated ten were cured.

The *treatment of recurrences* has been systematically carried out in the case of fifteen animals where the original treatment was begun on the second day. Five of these were ultimately cured. The details are given in Table IX.

TABLE IX.

Serial Number of Mouse.	Original Treatment.	Subsequent History and Treatment.
Y 94 . .	Acetyl-derivative, 1 : 40 on the second, third, fourth, and eighth days after inoculation.	First recurrence, 18 days after last injection, + trypanosomes in blood; acetyl-derivative, 1 : 40. Second recurrence, 20 days later, + trypanosomes in blood; atoxyl-derivative, No. 331, 1 : 40. Two days later, do. Third recurrence, 22 days later, + + + trypanosomes in blood; acetyl-derivative, 1 : 40. Three days later, atoxyl-derivative, No. 331, 1 : 40. Cured.
Y 106 . .	Acetyl-derivative, 1 : 40 on the second, third, eighth, and twenty-first days; trypan-blue, 1 : 200 on the fourth day.	+ + + Trypanosomes in blood 10 days after the last injection; acetyl-derivative, 1 : 40. Do. repeated 1, 2, 8, and 14 days later. Cured.
Y 107 . .	Acetyl-derivative, 1 : 40 on the second, third, and sixteenth days; trypan-blue, 1 : 200 on the fourth day.	+ + Trypanosomes in blood 21 days after last injection; acetyl-derivative, 1 : 40. Do. repeated 1 and 3 days later. Cured.
Z 69 . .	Acetyl-derivative, 1 : 40 on the second, third, fourth, and eighth day.	+ Trypanosomes in blood 2 days after last injection; acetyl-derivative, 1 : 40. + + + trypanosomes in blood next day, 1 : 200. Cured.
Z 77 . .	Acetyl-derivative, 1 : 40 on the second, third, fourth, and eighth days.	+ Trypanosomes in blood one day after last injection; acetyl-derivative, 1 : 40. + + + trypanosomes in blood next day, 1 : 200. Cured.

Specially worthy of note are—(1) Y 94, where cure took place after three recurrences, the whole treatment being with para-amido-phenylarsenic-acid-derivatives: (2) Z 69 and 77, where an atoxyl-resistant strain (see below) was present, and which were cured by a single injection of trypan-blue when abundant parasites were present in the blood; a result which would point to a special sensitiveness of the parasites to the latter compound under these circumstances.

II. The Biological Accommodation of Trypanosomes to Chemo-Therapeutic Agents.

Franke and Röhl (1907), working in Ehrlich's laboratory, settled the question why an animal infected with trypanosomes could ultimately succumb to a recurrence, in spite of treatment with a drug which, although it had not effected destruction of all the parasites, nevertheless had been effective earlier in the course of the infection in causing temporary disappearance of the trypanosomes. Working with mice infected with nagana and treated by parafuchsin-feeding, Franke found that when the recurrence ceased to respond to the treatment, inoculation into fresh animals gave rise to an infection which was from the very beginning uninfluenced by parafuchsin, administered either by feeding or by injection. Röhl so increased the "resistance" of this strain that on inoculation into animals fed with parafuchsin for days or even weeks beforehand, the infection proceeded as in normal untreated animals. As has been seen, animals similarly treated and then inoculated with the normal nagana-strain in 90 per cent. of cases never become infected.

Atoxyl-resistant strains of trypanosomes were developed by the author in a similar fashion. Mice were inoculated with the normal nagana-strain. On the appearance of scanty parasites in their blood the ordinary food was replaced by atoxyl-cakes. Thereafter, the trypanosomes usually persist in the blood, and may even increase in numbers for several days, and then disappear more or less suddenly. When the blood was found free of parasites on three or four successive days, ordinary food was again given. Daily examination of the blood was continued, and on the first appearance of trypanosomes atoxyl-feeding was again carried out as before. After several recurrences fresh animals were subinoculated, and the same proceeding again carried out. Subsequent to the third passage the subinoculation was, in general, made as soon as the infected animal had abundant parasites in its blood. From the twentieth passage onward one animal in each passage was fed with atoxyl from the time of inoculation, and the other twenty-four hours later. The strain was in this way carried through upwards of 150 passages. The results presented marked irregularities, and it became manifest that, in contrast to what holds in the case of the parafuchsin-resistant strain, absolute

resistance to atoxyl, as tested by means of feeding, could not be attained. Accordingly, the behaviour of the strain was tested by inoculating normally fed mice, and then, twenty-four hours later, when scanty parasites were present in the blood, injecting a full therapeutic dose of the acetyl- or the para-oxybenzylidene-derivative. This was done for the first time in the twenty-fourth passage. The result was that the infection was not protracted for even twenty-four hours. This is shown in Table X., which also illustrates the interesting fact that although atoxyl-feeding is much inferior to a therapeutic

TABLE X.

A.		*B.*	*C.**		*D*	
	Inoculation.	Inoculation.		Inoculation.		Inoculation.
First day	+[1]	+[1]	First day	...	First day	+
Second ,,	+	+	Second ,,	...	Second ,,	+ +
Third ,,	+ + +	+ + +	Third ,,	Inocul.	Fourth ,,	+ + + [2]
Fourth ,,	Dead	Dead	Fourth ,,	+	Fifth ,,	
			Fifth ,,	+ + [2]	Seventh ,,	−
			Seventh ,,	+ + +	Eighth ,,	
			Eighth ,,	−	Tenth ,,	−
			Tenth ,,		Eleventh ,,	+
			Eleventh ,,		Twelfth ,,	+ + + [2]
			Thirteenth ,,	−	Thirteenth ,,	Dead
			Fourteenth ,,	+		
			Fifteenth ,,	+ + + [2]		
			Sixteenth ,,	Dead		

[1] = 1 : 30 of the para-oxybenzylidene-compound injected subcutaneously.

[2] Atoxyl-feeding (indicated by the braced line).

* This animal was fed with atoxyl for two days prior to inoculation. All the mice were inoculated at the same time.

injection of the two para-amido-phenylarsenic-acid-derivatives in the treatment of an infection with the normal strain, nevertheless feeding frequently causes the resistant parasites to disappear from the blood for a time; but no cure as the result of atoxyl-feeding has occurred since the thirteenth passage. Further, the resistance is so highly developed that a full dose of either of the derivatives injected at the time of inoculation, into a different area of course, and then repeated twenty-four hours later, scarcely leads to forty-eight hours' protraction of the infection. Even after the resistance, as tested by injection of the para-amido-phenylarsenic-acid-derivatives, was fully developed, the results of atoxyl-feeding showed variations which are to be ascribed to individual peculiarities, as well as to mere differences in appetite. Thus, where feeding was begun at the time of inoculation, or even two days beforehand, *e.g.* mouse *C*, Table X., parasites usually were found in the blood after twenty-four to forty-eight hours. They then increased in numbers, and the animal might die as if untreated, or the trypanosomes might persist in enormous numbers and death be delayed for some

days, or they disappeared later. More rarely parasites did not appear so long as atoxyl-feeding was continued. Sometimes during feeding the parasites disappeared and then reappeared, although the feeding had not been discontinued, and so a chronic course resulted. A resistant strain derived from the above-mentioned "fed strain" was maintained by injection of 1 : 40 of one of the derivatives simultaneously with inoculation in each passage. The course of infection in untreated controls in upwards of 100 passages showed that the virulence of the trypanosomes remained unimpaired.

The following result shows that, under certain circumstances, atoxyl-resistance may be fairly rapidly acquired by trypanosomes. A mouse in the last stages of a nagana-infection—two out of four untreated controls were already dead—received a full therapeutic dose of a para-amido-phenylarsenic-acid-derivative. Next day this was repeated. On the day following atoxyl-feeding was begun, and was continued for eight days. Two days thereafter parasites were present in scanty numbers in the blood; accordingly, 1 : 40 of the acetyl-derivative was injected, but without effect, as the trypanosomes were abundant next day. A subinoculation was made, and from that a series of mice was inoculated, and the behaviour of the parasites toward 1 : 40 of the acetyl-derivative tested, as shown in Table XI

TABLE XI.

	A.	*B.*	*C.*	*D.*	Controls.	
	Inoculation.[1]	Inoculation.[1]	Inoculation.	Inoculation.	Inoculation.	Inoculation.
1 day	−	−	+[1]	+[1]	+	+
2 ,,	−	−	−	+	+ + +	+ + +
3 ,,	+	+	−	+	Dead	+ + +
4 ,,	+ + +	+ + +	−	+ + +	...	Dead
5 ,,	Dead	+ + +	−	+ + +	...	...
6 ,,	...	Dead	−	Dead	...	...
7 ,,	...	...	+	...	...	...
8 ,,	...	...	+ +	...	...	...
9 ,,	...	...	+ + +	...	...	...
10 ,,	...	...	Dead	...	...	...

[1] = Acetyl-para-amido-phenylarsinic-acid, 1 : 40 injected.

Thus, after ten days of treatment a distinct degree of resistance was present; but the behaviour of mouse *C*, where the blood was rendered free for five days, shows that the resistance was not sufficiently high to prevent a degree of therapeutic effect in certain individuals; the

strain was *semi-resistant.* In contrast to this is the mouse Y 94 (Table IX.) already referred to. Thus, while chemo-resistance is a property of the trypanosomes themselves, it is not unlikely that conditions for the development of such resistance may vary with the particular host.

A strain resistant to trypan-blue was readily obtained. Starting with an injection of 1 : 600—the dye is not well resorbed from the alimentary canal—and gradually increasing the dose in subsequent passages, it was found that after four weeks, in the tenth passage, the therapeutic dose (1 : 200) under standard conditions, caused no protraction of the infection, and a week later the same dose injected at the time of inoculation was tested, with the same result.

The original nagana-strain was somewhat susceptible to *trypan-red* (trypan-red debilis—Ehrlich), as the therapeutic dose under standard conditions rendered the blood free for two days, and delayed death for four days beyond the controls. Proceeding as in the case of the trypan-blue-resistant strain, complete resistance to a dose of 1 : 200 injected at the time of inoculation was attained in three weeks.

It has been possible to render a strain highly resistant both to the para-amido-phenylarsenic-acid-derivatives and to trypan-blue. Animals inoculated with the atoxyl-resistant strain were treated with increasing doses of trypan-blue in successive passages, just as described above. After five weeks the resistance to trypan-blue was complete. Up to the forty-first passage the treatment was only with trypan-blue. As tests in the twenty-seventh and forty-first passages showed (Table XII.), the resistance to the acetyl-derivative persisted unimpaired,

TABLE XII.—*Twenty-seventh Passage.*

	A. Inoculation: at the same time— Acetyl-derivative, 1:40. + Trypan-blue, 1 : 200.	*B.* Inoculation: at the same time— Trypan-blue, 1 : 200.	Control. Inoculation.
First day after inoculation	–	–	–
Second ,, ,,	+	+ +	+ +
Third ,, ,,	+ + +	+ + +	+ + +
Fourth ,, ,,	Dead	Dead	Dead

Forty-first Passage.

Inoculation: at the same time—acetyl-derivative, 1 : 40

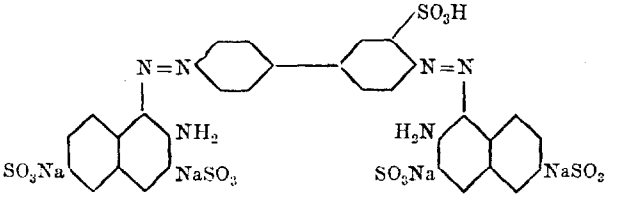
First day after inoculation	+	Trypan-blue, 1 : 200
Second	,,	+ + +
Third	,,	Dead

and an injection af a full dose of this substance at the time of inoculation, along with a full dose of trypan-blue administered simultaneously (twenty-seventh passage), or on the following day (forty-first passage), did not influence the infection. This strain was later on rendered resistant to parafuchsin in addition, so that ultimately it possessed a *triple resistance.*

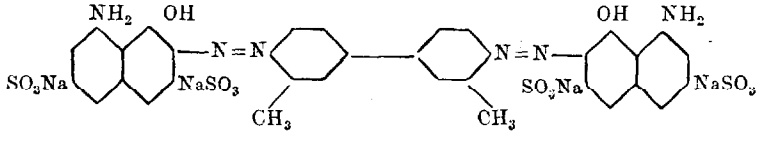
Thus, by subjecting a strain of trypanosomes in the body of an infected animal to the prolonged action of a chemo-therapeutic agent, in a manner insufficient to produce complete sterilisation, there can result a profound alteration in the biological character of the parasites, which in fresh animals will give rise to an infection that no longer responds to the compound in question. It is to be noted that the resistance, which may be described as histogenetic immunity, is relative, not absolute, as the results of atoxyl-feeding in the atoxyl-resistant strain show.

Further Properties of the Chemo-Resistant Strains.

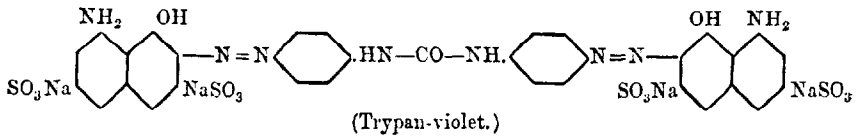
As has been seen, the atoxyl-resistant strain is resistant to the two para-amido-phenylarsenic-acid-derivatives. The parafuchsin-resistant strain is resistant to Döbner's violet. The trypan-blue-resistant strain is resistant to trypan-violet and to trypan-red, and the trypan-red-resistant strain is resistant to trypan-blue. The resistance, accordingly, implies not merely specific insusceptibility to the action of the compound in response to which it has been accquired, but also a positive increase of resistance, more or less marked, but always appreciable, toward substances of the same chemical type. This *group-resistance* in the case of the benzidin dyes is interesting in view of the differences in details of constitution of the three members tested. The only feature common to all is the presence of sulphonic radicals (HSO_3) in the positions 3, 6 in the naphthalin-nuclei of the side-chains.



(Trypan-red.)



(Trypan-blue.)



(Trypan-violet.)

Chemo-resistance finds a parallel in the serum-resistance of trypanosomes observed by Franke (1905), and of spirochætes (Levaditi and Roche (1907)). On the other hand, the development of resistance to compounds of one group has not been accompanied by a diminution in susceptibility to members of the other groups. Thus, the atoxyl-resistant strain was susceptible to trypan-blue, trypan-red, trypan-violet, parafuchsin, ethyl-violet and Döbner's violet, and in the same way the trypan-blue-resistant strain and the parafuchsin-resistant strain were susceptible to all the compounds of the other groups. The development of specific chemo-resistance, as well as the natural variations in susceptibility of different species of trypanosomes toward particular substances, recorded by various workers (Ehrlich, Mesnil and Nicolle, Wenyon (1907)), and the alterations in susceptibility of the same trypanosome-species as the result of passages through different animals (Franke (1905)), are unintelligible apart from Ehrlich's theory that a direct chemical relationship exists between the therapeutic agent and particular atom-groups of the protoplasm of the parasites, the *chemo-receptors*, a conception which has been arrived at independently by Langley (1906) in another field of investigation.

Chemo-resistance when once acquired persists for long periods when the strains are passed through normal untreated animals. Thus, one atoxyl-resistant strain was still highly resistant after 140 passages through mice during fourteen months. In another strain the resistance was still marked after 67 passages during six months, but in the eighty-ninth passage, seven weeks later, the resistance was gone. The trypan-blue-resistant strain was still highly resistant after eighty passages extending over six months. So also, after five passages through rabbits over a period of five months, the high specific resistance to trypan-blue was still retained, as tested by treating mice inoculated with blood from the last rabbit.

Inoculations with mixtures of two resistant strains show that the two varieties, although derived from the same stock originally, behave as if they were distinct species.

Thus, a series was inoculated with a mixture of blood from a mouse infected with the atoxyl-resistant strain, and from one infected with the parafuchsin-resistant strain. Half of the animals were treated with parafuchsin under standard conditions. In these the disease proceeded without check. From one of these, on the day after treatment, a number of mice were subinoculated. Part of these were treated with parafuchsin, which had no effect; while the rest, treated with acetyl-para-amido-phenylarsenic-acid, were cured. The other half of the animals of the original mixed infection were treated with the acetyl-derivative, but without effect. Subinoculations were treated as before; but in this instance only those injected with parafuchsin responded to treatment.

After such a mixed infection has been repeatedly passed through normal animals the two resistant components may still be present side by side on testing, as described above. This was the case with the mixture of the trypan-blue resistant and the atoxyl-resistant strains after twenty-five passages; but in the case of the parafuchsin-resistant and the atoxyl-resistant strains the former element was still evident in the eleventh passage, but in the twenty-fifth had disappeared, as shown in Table XIII. This is probably to be explained by the atoxyl-resistant component outgrowing the other, and so leading to its elimination.

TABLE XIII.

	Treatment with Parafuchsin.					Treatment with the Acetyl-Derivative.		Controls.	
11th Passage. 1 day after inoculation.	Inf. +1 : 1000	Inf. +1 : 1200	..	..	..	Inf. +1 : 40	Inf. +1 : 40	Inf. ÷	Inf. +
2 ,,	−	−	..	..	..	++	+	+++	++
3 ,,	−	+	..	..	..	+++	+++	Dead	Dead
4 ,,	+	+	..	..	..	Dead	Dead	..	..
5 ,,	++	++	..	..	..	..	..	..	..
6 ,,	+++	Dead	..	..	..	..	..	..	..
7 ,,	Dead	..	..	..	..	..	..	..	..
25th Passage. 1 day after inoculation.	Inf. +1 : 1000	Inf. +1 : 1000	Inf. +1 : 1200	Inf. +1 : 1200	Inf. +1 : 1200	Inf. +1 : 40	Inf. +1 : 40	Inf. +	Inf. +
2 ,,	−	−	−	+	+	+	+	+	+
3 ,,	−	−	+	−	−	+++	+++	+++	+++
4 ,,	−	−	+	−	−	Dead	Dead	Dead	Dead
5 ,,	−	−	−	−	−				
6 ,,	−	−	−	−	−				
7 ,,	−	−	−	−	−				
8 ,,	−	−	−	−	−				
9 ,,	−	−	+	−	−				
10 ,,	−	−	+++	−	−				
11 ,,	+	−	Dead	−	−				
12 ,,	++	−	..	−	+				
13 ,,	+++	−	..	−	++				
14 ,,	Dead	−	..	−	+++				
15 ,,	..	−	..	−	Dead				
16 ,,	..	−	..	+	..				
17 ,,	..	−	..	++	..				
18 ,,	..	−	..	+++	..				
19 ,,	..	−	..	Dead	..				
150 ,,	..	Cured	..	..	..				

CONTROL.—Parafuchsin-resistant strain; pure culture, through untreated animals.

	Treatment with Parafuchsin.		Controls.	
25th Passage. 1 day after inoculation	Inf. +1 : 1000	Inf. +1 : 1200	Inf. ÷	Inf. +
2 ,,	+++	++	++	++
3 ,,	+++	+++	Dead	Dead
4 ,,	Dead	Dead	..	..

III. Immunity-Phenomena.

The uniform high susceptibility of mice to infection with the nagana-strain is shown by the acute and regular course of the experimental disease. Accordingly, prolongation of the incubation-period, or protraction of the course of infection, are certain indications of immunity on the part of the mouse, or of attenuation of the trypanosomes.

1. *The behaviour of the Trypanosomes immediately after the administration of a Therapeutic Agent.*

Twenty-four hours after the injection of a therapeutic dose of Ehrlich's para-amido-phenylarsenic-acid-derivatives no parasites are found in the blood on microscopic examination. Accordingly, for the study of immunity phenomena the animal must be reinoculated. But where smaller doses are employed, or the drug used is less efficient, *e.g.* atoxyl, the trypanosomes may not disappear for several days. This suggests that anti-bodies have developed as the result of destruction and resorption of a proportion of the parasites, and have then come into play (Ehrlich and Shiga). This phenomenon has been very pronounced in experiments with ethyl-violet and Döbner's violet, the parasites not merely persisting for some days, but, in addition, increasing greatly in numbers before disappearing (see Table XIV.).

Table XIV.

	A.	B.	Control.
	Inoculation.	Inoculation.	Inoculation.
1 day after inoculation .	+ Ethyl-violet, 1 : 1500	+ Döbner's violet, 1 : 750	+
2 ,, ,, .	+ +	+ +	+ +
3 ,, ,, .	+	+ +	Dead
4 ,, ,, .	+	−	...
5 ,, ,, .	−	−	...
6 ,, ,, .	−	−	...
7 ,, ,, .	−	+	...
8 ,, ,, .	+	+ + +	...
9 ,, ,, .	+ + +	Dead	...
10 ,, ,, .	+ + +	...	...
11 ,, ,, .	Dead	...	...

2. *Incomplete destruction of the Trypanosomes, followed by Recurrence.*

Table XV. shows details of the recurrences in a series of therapeutic experiments made by Ehrlich and the author, to test the efficiency of various para-amido-phenylarsenic-acid-derivatives in nagana-infections under standard conditions. For the most part the therapeutic dose was used; but in some instances, where the compounds were fairly efficient, smaller doses were also employed. Those cases are neglected where the blood was not rendered free of parasites for at least three days. It will be observed that nearly three-fourths of the 138 recurrences occurred between the sixth and eleventh day. In only four, less than 3 per cent., did the recurrence take place after the fifteenth day. When the parasites reappeared in the blood, death followed in over 90 per cent. within three days, just as occurs in the infection in untreated animals. Six days was the longest time between reappearance of the trypanosomes and death. The conclusions are—(1) where destruction of the parasites is not complete the reappearance of trypanosomes is seldom much delayed beyond the end of the immunity-period, as determined by reinoculation experiments, which averages ten days in length (see below, p. 186); (2) the virulence of the residual parasites is practically unaffected.

In the case of infections treated on the second day by a single dose of the acetyl- or the para-oxybenzylidene-derivative, it was found that the period during which the blood remained free from parasites was longest with the largest doses. Further, the length of the free period after the injection of the therapeutic doses of these compounds on the second day was on the average fourteen days, as compared with nine days in the series treated on the first day (Table XV.).

TABLE XV.

Day following the therapeutic injection	4	5	6	7	8	9	10	11	12	13	Later than the twentieth day	
				14	15	16	17	18	19	20		
Total number of recurrences on each day	3	7	20	17	17	32	17	7	6	4	None	
				3	1	0	2	0	0	2		
Percentage of recurrences on each day	2·2	5·1	14·5	12·3	12·3	23·2	12·3	5·1	4·3	3·0	0	
				2·2	0·7	0	0·15	0	0	0·15		

Where the original infection or a recurrence has been treated by *repeated injections* the free period has frequently been much longer. Thus, in one mouse infected with nagana, and treated throughout with para-amido-phenylarsenic-acid-derivatives a second recurrence took place fifty-three days after the first, which had been treated by repeated injections, the last of which was given thirty-nine days before the second recurrence. The preservation of virulence by the trypanosomes is well illustrated by a case where the second recurrence, sixty-

nine days after the original inoculation and forty-one days after the first recurrence, which had been treated with four therapeutic doses and atoxyl-feeding for nine days was left untreated, and the mouse succumbed to the disease four days later.

The condition of "*Immunitas non sterilisans*" (Ehrlich), where attacks of the disease and immunity-periods alternate, or where an equilibrium is established between parasite and host, so that the latter continually has parasites in its blood but suffers no ill effect, is frequent in diseases due to protozoa. In chemo-therapeutic experiments comprising over 1000 mice infected with nagana the first recurrence, when untreated, has quickly terminated in death. In only four animals has a chronic course resulted after treatment; the compound used being an impure basic dye, whose active constituent has not been isolated, either alone or with one of the para-amido-phenylarsenic-acid-derivatives. The parasites appeared in the blood at irregular intervals, and followed no regular course either as regards their numbers or the length of time for which they persisted. Subinoculations were made from one of these, and then further passages. The strain was markedly diminished in its virulence, which had not risen to normal till the tenth passage. It is interesting that one mouse of the *second* passage, after an incubation-period which was not much protracted (two days), developed a typical chronic infection (Table XVI.). Death occurred on the 128th day, with abundant parasites in the blood. The liver and spleen were much enlarged, weighing respectively 4·8 and 2·1 grms. It is evident that this animal was exceptionally resistant, and that the infection with an attenuated strain brought into evidence this individual characteristic, which would never have appeared had the inoculation been made with the ordinary highly virulent strain.

TABLE XVI.

	Inoculated.				
1 day	−	56 day	+	105 day	5 days
2 ,,	−	57 ,,	+	109 ,,	4 examinations
3 ,,	+	58 ,,	+ +		−
5 ,,	+ +	59 ,,	+	110 ,,	+
6 ,,	+ +	60 ,,	18 days	111 ,,	+
7 ,,	+	77 ,,	9 examinations	112 ,,	+ +
8 ,,	+		−	113 ,,	Examined daily
9 ,,	+	78 ,,	+	115 ,,	+ + +
10 ,,	+	79 ,,	21 days	116 ,,	+ +
11 ,,	+	99 ,,	16 examinations	117 ,,	6 days
12 ,,	+ +		−	122 ,,	5 examinations
13 ,,	+ + +	100 ,,	+		+ + +
14 ,,	+ + +	101 ,,	+	123 ,,	+ +
15 ,,	+	102 ,,	+ + +	124 ,,	+ + +
16 ,,	40 days	103 ,,	+ + +	127 ,,	+ + +
55 ,,	28 examinations	104 ,,	+ +	128 ,,	Dead
	−				

3. *Chronic Infections following Inoculation with Strains which have been treated* in vitro.

Defibrinated trypanosome-containing blood was mixed with varying strengths of parafuchsin. The parafuchsin solution was made by diluting a 1 per cent. solution of the dye in methyl alcohol with sterile isotonic (8 per cent.) watery cane-sugar solution, as salt tends to precipitate the dye. After the mixture had stood in the laboratory for twenty minutes, mice were inoculated with the mixture, and received the usual dose of parasites. Controls were, of course, always made with suspensions of the infective blood in sugar-solution without any dye, and showed that the sugar itself had no injurious effect on the trypanosomes under these conditions. So also, the amount of alcohol from the stock-solution of the dye was not a disturbing factor. In the case of two mice in one series, a dilution of 1 : 300,000 of parafuchsin caused the trypanosomes to appear three days later than in the controls, thus showing how delicate the biological test is as a means of detecting damage of the parasites. Of thirty-six animals inoculated with trypanosomes which had been acted on by parafuchsin in dilutions varying between 1 : 80,000 and 1 : 9000, eight became subjects of a chronic infection. The longest duration of the disease was 114 days, parasites having been first observed on the fifteenth day. Five mice never became infected. The varying behaviour of individuals is striking. Thus, two mice were inoculated at the same time with equal amounts of infective blood, treated with parafuchsin 1 : 18,000. Mouse No. 1 had scanty parasites in its blood six days later, and died of the infection two days afterwards Mouse No. 2 never became infected. In another series, two mice were inoculated with blood treated with parafuchsin 1 : 50,000. Mouse No. 1 had parasites in its blood for the first time nineteen days later; the infection ran a chronic course, and death occurred on the thirty-sixth day after inoculation. Mouse No. 2 had trypanosomes in its blood for the first time on the eleventh day, and it succumbed to the infection two days later. In all the chronic infections death occurred with abundant parasites in the blood. It is remarkable that such semi-immunity, analogous to what Koch produced by inoculating cattle with attenuated trypanosomes, and Franke observed on reinoculating a rabbit which had been cured of an infection with mal de Caderas by injections of trypan-red and arsenic, occurred so frequently where the trypanosomes were acted on *in vitro*, but were extremely rare in infections treated by injections.

4. *Reinoculation Experiments.*

(*a*) *Animals the subjects of chronic infection.*—Four such animals were reinoculated with the usual amount of blood containing the normal virulent nagana-parasites. Of these, one mouse, which had

become the subject of a chronic infection as the result of treatment with the impure basic dye, died at the same time as the controls. The other three were animals which had been injected with trypanosomes of the virulent nagana-strain which had been acted on by parafuchsin *in vitro*. Two died of the infection five days after inoculation—that is, two days after the controls. The third mouse, which had been inoculated with trypanosomes acted on by parafuchsin 1 : 36,000, showed no parasites in its blood for fourteen days after reinoculation with the virulent strain. It was, accordingly, once more inoculated, and thereupon infection developed promptly, and it died at the same time as the controls. These results are similar to observations of Martini (1905) in the case of horses.

(*b*) *Reinoculation of animals previously infected and treated by a chemo-therapeutic agent.*—As Ehrlich has pointed out, reinoculation with the virulent strain is the most precise method of determining the duration and degree of the immunity following treatment with a chemo-therapeutic agent. By adopting such a proceeding both the virulence and the amount of the infective material are known, and accordingly any abnormality in the development of the infection can be due only to the action of anti-bodies. Ehrlich and the author have in this way determined the duration of the immunity-period in the case of the very virulent nagana-strains. Animals were inoculated with, for example, the parafuchsin-resistant strain, and twenty-four hours later, scanty parasites being present in the blood, a therapeutic dose of the acetyl- or the para-oxybenzylidene-derivative was injected; then, after four or five days the animals were reinoculated with the parafuchsin-resistant strain, the normal strain, and the atoxyl-resistant strain, etc. Two series of controls were made along with the reinoculations — (1) "*treated*" *controls*: animals which had been injected with the same dose of the derivative, and at the same time as the infected mice, and which show that the preventive action of the drug had disappeared; (2) *ordinary normal animals.* The details of such a series are given in Table XVII.

The results show—1. That with these extremely virulent nagana-strains the immunity-period is of not more than ten days' duration after the therapeutic injection; at the end of this time the parasites appear in the blood, and death quickly follows. 2. The immunity-period is most clearly demonstrated where the reinoculation has been made with the same variety of the nagana-strain as that used for the primary infection; that is, the parafuchsin-resistant strain in the series shown in Table XVII. In the case of reinoculation with the atoxyl-resistant strain no immunity can be shown to exist at all.

Under conditions still more favourable for the appearance of immunity, namely, when treatment was begun on the second day, at a time when abundant parasites were present in the blood, and the therapeutic injection was repeated, it was clearly apparent that no

immunity existed on reinoculation with the atoxyl-resistant strain. Seven animals were inoculated with the original nagana-strain; forty-eight hours later, abundant parasites being present in the blood, they were injected with 1 : 40 of the acetyl-derivative. The treatment was repeated on the following day. Two days after the second injection three of the mice were reinoculated with the original nagana-strain, and four were inoculated with the atoxyl-resistant strain. In all those reinoculated with the original strain the blood remained five days free of parasites, while in the case of the second inoculation with the atoxyl-resistant strain parasites were present in the blood twenty-four hours later, and all the animals died at the same time as the controls.

TABLE XVII.

	Inf. P-R.S.	Inf. P-R.S.	Inf. P-R.S.	"Treated" Controls.			Untreated Controls.		
1 day	+[1]	+[1]	+[1]	1	1	1	..	..	..
2 „	-	-	-	..	..	..	..	..	..
3 „	-	-	-	..	..	..	..	..	..
4 „	-	-	-	..	..	..	..	..	..
5 „	-	-	-	..	..	..	..	..	..
6 „	- Inf. P-R.S.	- Inf. O.S.	- Inf. A-R.S.	Inf. P-R.S.	Inf. O.S.	Inf. A-R.S.	Inf. P-R.S.	Inf. O.S.	Inf. A-R.S.
7 „	-	+	+	+	+	+	+	+	+
8 „	-	+	+++	+	+++	+++	++	+++	+++
9 „	-	+++	Dead	+++	Dead	Dead	+++	Dead	Dead
10 „	-	+++	..	Dead	..	..	Dead	..	..
11 „	+	Dead	..	..	..	..	..	..	..
12 „	+++	..	..	..	..	..	..	..	..
13 „	Dead	..	..	..	..	..	..	..	..

P-R.S. = Parafuchsin-resistant strain.
A-R.S. = Atoxyl-resistant strain.
O.S. = Original nagana-strain.

[1] = Injection of the para-oxybenzylidene-derivative, 1 : 45.

These results, taken together with the experiments on the behaviour of infections with mixtures of the resistant strains when treated with a chemo-therapeutic agent toward which one of the components is resistant, show that from the point of view of the immunity-reaction the different modified nagana-strains behave as if they were distinct species.

GENERAL CONCLUSIONS ON IMMUNITY PHENOMENA.

1. Where the trypanosomes are destroyed *in vivo* through the influence of chemo-therapeutic agents an active immunity results.

2. The immunity which follows treatment with a single therapeutic dose of para-amido-phenylarsenic-acid or the para-oxybenzylidene-deriv-

ative, injected twenty-four hours after inoculation, when scanty parasites are present in the blood, as tested by reinoculation with the same virulent nagana-strain as was used for the primary infection, does not last more than ten days.

3. Where the treatment has not effected complete sterilisation the existence of immunity is also apparent. In such cases a recurrence takes place after the disappearance of the immunity. As a rule, the trypanosomes reappear about the tenth day after the therapeutic injection, just as happens in the reinoculation-experiments.

4. In those instances where treatment is begun on the second day, when the parasites are present in large numbers in the blood, the resulting immunity appears to be of longer duration (a greater "Ictus immunisatorius"). Thus, where sterilisation is not complete the recurrence takes place later, and has been observed twenty-two days after treatment by a single injection.

5. When once the trypanosomes have reappeared in the blood they rapidly increase in numbers, and death occurs within three to four days. An exception to this rule, in the form of a chronic infection, is a very rare occurrence.

6. The immunity which results after treatment (by the acetyl- or the para-oxybenzylidene-amido-phenylarsenic-acid-derivative) of an animal infected with one of the modified nagana-strains, *e.g.* the para-fuchsin-resistant strain, is specific in so far that it is effective against reinoculation with the same strain; but not against the atoxyl-resistants train. Thus, it appears that the immunity-reaction is affected by very delicate biological differences. This result leads to the conclusion which is in agreement with Koch's view (1904), that differences in the immunity-reaction are not a sufficient basis for the classification of different species of trypanosomes.

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